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Replicative Life-Span of Cultivated Human Cells

Effects of Donor's Age, Tissue, and Genotype

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In vitro studies of the longevity of fibroblast-like human diploid somatic cells in over 100 mass cultures and 200 clones from a variety of donors have provided evidence for a limited replicative life-span, a negative regression of growth potential on age of donor from the 1st to the 9th decades, and a variable growth potential as a function of the tissue of origin. The results lend support to the hypothesis that the limited growth potential of cultured somatic cells is a manifestation of senescence at the cellular level. A striking diminution of the growth potential of cultures from patients with Werner's syndrome, a hereditary disease manifested by early and widespread degenerative changes, was also observed.

Additional key words: Aging, Diabetes mellitus, Fibroblast cultures, Progeria, Rothmund's syndrome, Werner's syndrome.

Hayflick[10] described three stages in the natural history of cultivated human diploid "fibroblast" cultures derived from various somatic tissues. Stage I is the establishment of the cultures; stage II consists of reproducible cycles of growth throughout successive passages; and stage III comprises progressive increases in population doubling times, with eventual failure of cell replication. Hayflick suggested that this restricted growth potential is a manifestation of senescence at the cellular level. We now report experiments which lend support to this interpretation. The longevity of skin fibroblast cultures has been found to be inversely correlated with the age of the donor, confirming and extending the results of Hayflick[9-11] and of Goldstein, Littlefield, and Soeldner.[7] Furthermore, a specific autosomal recessive mutation—that responsible for the extensive degenerative pathology associated with Werner's syndrome—results in a sharply limited *in vitro* replicative life-span, confirming and extending earlier work from this laboratory.[3, 18] Finally, we emphasize the importance of standardizing the site of biopsy for *in vitro* studies on cell senescence because the replicative life-span of the cultures has also been found to be a function of the tissue of origin, and was first suggested by the work of Swim and Parker.[30]

MATERIALS AND METHODS

Tissue specimens were diced into 1-mm. cubes, and replicate cultures consisting of two such cubes per Leighton tube (Bellco Glass, Inc., Vineland, New Jersey) were established. The cubes of tissue were sandwiched between a glass slide (cut to fit the Leighton tube) and the bottom of the tube. Two milliliters of Waymouth's medium prepared in this laboratory with 9.0 per cent heat-inactivated newborn calf serum and penicillin (50 units per ml.) were added, and the tubes were incubated at 37° C. in an atmosphere of 5 per cent CO_2 in air. The medium was changed once during the 1st week, then twice weekly until approximately 40 per cent of the glass surface was covered with fibroblasts. At that time they were trypsinized,[15] and the 30,000 to 100,000 cells harvested were transferred to a 4-ounce prescription bottle. Upon confluency, the cells were again trypsinized and were transferred to 6-ounce prescription bottles. Repeated trypsinizations were conducted when the monolayers became partially confluent, usually every 6 to 8 days during stage II, at which time cell counts ranged from 1×10^6 to 3.5×10^6. At each passage, the bottles were reseeded with 400,000 cells. Hemocytometer counts of unattached cells from randomly selected cultures of skin (16 to 20 hours after plating) indicated that 75 to 90 per cent of the plated cells attached to the glass, with somewhat fewer cells attaching during later passages than earlier passages. Cell counts performed with an electronic particle counter (Coulter Electronics Company, Hialeah, Florida) were deceptive in that increasing amounts of cytoplasmic debris, often in clumps, were encountered with later passages, yielding erroneously high "cell counts" and proportionately larger numbers of "unattached cells." The data to be presented are calculated on the basis of 100 per cent cell attachment and the assumption that all cells participate in the population doubling. Therefore, the total number of cell doublings calculated for any given culture is a minimal estimate. For example, in a typical passage during stage II, 7 days after an inoculum of 400,000 cells, 2.4×10^6 cells could be harvested by trypsinization. On the basis of the assumptions that all 400,000 cells survived the trypsinization and attached to the glass and that

all of these cells participated in the growth of the culture, we would calculate an expenditure of 2.5 cell doublings for that particular passage. However, if only 75 per cent of the plated cells attached to the glass and if only two-thirds of these cells subsequently underwent mitoses, an average of 3.5 cell doublings would have been required to achieve a population of 2.4×10^6 cells. A culture was terminated when, after 1 month, the total yield of cells in a given passage was less than 400,000. The cumulative number of cell doublings from all previous passages (beginning with the transfer from the Leighton tube to the 4-ounce prescription bottle) was then determined and utilized in the construction of Figures 1 and 2. Statistical analysis of the data was performed through the courtesy of Dr. Julian I. E. Hoffman; regression lines were calculated by the method of least squares.[29] Mycoplasma or other microorganisms could not be cultured from randomly selected monolayers and supernatant media obtained throughout the course of the study. The cultures for mycoplasma were performed aerobically and anaerobically by Dr. George E. Kenny with the use of a soy peptone dialysate broth medium.[12]

Subjects were selected at random from laboratory personnel and from inpatients and available autopsies with a wide variety of congenital anomalies, metabolic disorders, and cardiovascular, degenerative, and neoplastic disease. Subjects known or suspected of having an infectious disease were never used. Analysis of the data failed to reveal any relationship between the cumulative number of cell doublings achieved by a culture and the sex of the patient, or, in the case of autopsy specimens, the time after death at which a biopsy was obtained. Except for a rare instance of microbial contamination, cultures could be regularly established up to at least 72 hours postmortem (in the present study, however, nearly all of the postmortem biopsies were obtained within 24 hours after death). No difference in growth potential could be discerned between groups of antemortem and postmortem specimens.

Summaries of Clinical Histories

Werner's syndrome is an autosomal recessively inherited disease characterized by a wide variety of striking degenerative features which we have referred to as a "caricature" of normal aging.[3] The earliest manifestation of the disease is a symmetrical retardation of growth, followed by graying of the hair, atrophy of subcutaneous fat and skin, hyperkeratosis, generalized loss of hair, alterations in the voice, cataracts, skin ulcers, and, in approximately one-half of the cases, diabetes mellitus. Severe arteriosclerosis, osteoporosis, skeletal muscle atrophy, and severe testicular atrophy are typical. At least 10 per cent of the patients develop serious neoplasms during their average life-span of 47 years. Progeria (the Hutchinson-Gilford syndrome), possibly also autosomal recessive in inheritance, results in even more severe pathology, with signs of "senility" appearing during the 1st decade of life, death sometimes resulting from complications of coronary artery atherosclerosis within the first 2 decades of life.[3] Rothmund's syndrome is another recessively inherited disease which has sometimes been believed to be related to Werner's syndrome.[3] Cultures were established from three patients with Werner's syndrome, from a 4-year-old girl with progeria, and from a 2-year-old boy with Rothmund's syndrome. Abstracts of their case histories follow.

H. Mc. G. (University of Washington Hospital, 077-54). An extensive report of clinical, laboratory, and pathologic findings of this Japanese-American housewife appears as case 1 in Reference 3. She displayed typical signs and symptoms of Werner's syndrome, including growth retardation, cataracts (diagnosed at age 22), graying and loss of hair, glycosuria, skin atrophy, ulcers, osteoporosis, and voice changes. The cultures reported in the present study were established when the patient was age 49.

P. N. (National Institutes of Health, 06-16-12). This Caucasian man with Werner's syndrome is cited in the addendum of Reference 3. He first developed difficulty at age 36 when hip pain led to the discovery of a calcific deposit. At age 37 he developed recurrent ulcerations of both feet, primarily at pressure points; before this, however, the patient had observed that his skin was quite dry and easily bruised. Bilateral cataracts were diagnosed at age 38 and were surgically removed at age 41. He had had a high pitched voice throughout his life. Because of frequent sore throats and difficulties in swallowing, he was operated on at age 42 and was told that a papilloma was removed; at that time a diagnosis of diabetes mellitus was made. The patient was the father of two normal children; however, since approximately age 45, there was loss of libido and impotence. Of seven siblings, one sister had diabetes and cataracts, but none of the other manifestations of Werner's syndrome. The parents of the patient were not related. Physical examination at age 48 revealed a thin, balding man who appeared considerably older than his stated age. There was generalized loss of body hair and slender extremities. The skin was dry and thin and markedly atrophic over both legs and feet, the latter areas showing both hyper- and hypopigmentation. Large purulent ulcers were present on the posterior aspects of both heels, and several small punched-out ulcers were present on the toes and other bony prominences of the feet. There was a grade 2 blowing systolic murmur along the left sternal border. The prostate was diffusely enlarged, the testis descended and firm, and the penis small. Laboratory studies revealed a moderate iron deficiency anemia, an elevated fasting blood sugar (122 mg. per 100 ml.), and normal values for calcium, phosphorus, and alkaline phosphatase. The serum γ-globulin was elevated to 1.9 gm. per 100 ml. An electrocardiogram revealed diffuse osteoporosis of the pelvis, lumbar spine, legs, and feet; tendinous and soft tissue calcification was present in several areas of both feet. Intravenous pyelogram revealed bilateral hydronephrosis with hydroureters and bilateral distal ureteral obstruction. A perineal needle biopsy revealed undifferentiated carcinoma of the prostate. The patient was treated with bilateral orchiectomy followed by stil-

bestrol; sections of the testes revealed spermatogenic arrest and moderate interstitial sclerosis. Cultures were established from both skin and testis from this patient at age 48.

W. L. (National Institutes of Health, 07-00-63). This unmarried Caucasian man with Werner's syndrome was well until age 13 when his voice was noted to be scratchy and hoarse; at that time, he was also treated with horse gonadotrophins because of a small phallus. At age 14 he developed pain in the bottom of both feet whenever he put weight on them, and calluses were noted to be present. At age 24 he developed an ulcer on the lateral aspect of his left heel; shortly thereafter bilateral cataracts were diagnosed. After developing a second heel ulcer at age 28, a diagnosis of Werner's syndrome was made. At age 31 he was found to have diabetes mellitus. No other members of his family had any stigmata of Werner's syndrome; however, it is of interest that the parents of the patient were first cousins. On physical examination at age 37, he appeared 20 years older. The hair was gray, the extremities thin and wasted, and there were flexion contractures of both knees. A grade 2 systolic murmur was noted along the left sternal border. The penis was small and the testes soft, measuring approximately 2 by 2 by 2.5 cm. Skin of the extremities was dry and taut, with several small ulcers on the feet, and large ulcers exposing the gastrocnemius tendons on the posterior aspects of both feet. There were hyperkeratoses over both elbows. Pubic and axillary hair was sparce, but a grayish beard was noted. X-rays revealed diffuse vascular calcifications and diffuse osteoporosis especially in the legs and feet, and soft tissue calcifications of the knees, feet, and ankles. The 2-hour postprandial blood sugar was elevated and was followed by hypoglycemia at 5 hours. Skin cultures were established at age 37.

M. M. (University of Washington Hospital, 509-86). This Caucasian girl was referred because of growth failure, noted only after the 1st year of life. Although at birth she weighed 8 pounds 6 ounces and was 21 inches long, she weighed only 19 pounds 11 ounces and was only 30¾ inches long at 28 months. At approximately age 2, her hair began to fall out, her nails were noted to be thin and fragile, strabismus developed, and the veins of the scalp appeared to be quite prominent. Except for weakness and some fatigue, the patient seemed to be otherwise in good health and only rarely suffered from infections. Her parents (unrelated) were age 27 and of normal stature, as were the maternal and paternal grandparents; three female siblings were living and well. When examined at age 38 months, she weighed 21.9 pounds and was 33.1 inches tall. On physical examination, she had the appearance of a thin and tiny old lady. The skin was very thin, and there was striking atrophy of subcutaneous fat—especially over the scalp. The nose appeared "beaked." The nails were thin and furrowed. Scalp hair was very blonde and extremely sparse. There were no cataracts, skin ulcers, or hyperkeratoses, and there was no evidence of diabetes. The serum cholesterol varied from 168 to 213 mg. per 100 ml. and the serum triglycerides, from 82 to 252 mg. per 100 ml. A diagnosis of progeria was made by Professor David W. Smith and his colleagues, to whom we are grateful for the opportunity of evaluating this patient. Skin fibroblast cultures were established when the patient was 58 months old.

G. A. (University of Washington Hospital, 747-08). This Caucasian boy was seen at age 34 months, with a chief complaint of peculiar patterns of skin pigmentation with areas of scarring. Except for a scaly red rash over the forehead, the child's skin had been clear (but pale) until age 6 to 7 months, at which time a reticular macular facial hyperpigmentation was noted, gradually also involving arms, legs, and buttocks, with areas of hypopigmentation, hyperpigmentation, flakiness, drying, and scarring. The unrelated parents were age 25, and there was a normal male sibling; no similar pathology had been observed in former generations of the pedigree. On physical examination, the small but symmetrically developed child was 85 cm. tall and weighed 10.66 kg. There was a broad (1 to 10 mm.) reticular network of hyperpigmentation over the face, neck, arms, legs, and buttocks, with circular areas of depigmentation of the thighs and buttocks. The skin of the extremities was dry, scaly, and focally hyperkeratotic. Scattered small telangiectases were noted on the cheeks. There were no eyebrows and only two eyelashes; scalp hair was blond, dry, abundant, and fine. Subcutaneous tissue was present over all areas of the body. There was no tightening or ulceration of the skin. An ophthalmologist had prescribed glasses for a refractive error, but cataracts were not reported. The right thumb (including the first metacarpal) was congenitally absent, and the left thumb was hypoplastic. There was a proximal fusion of the radius and ulna bilaterally; the secondary ossification centers of L4 had not formed. The Dermatology, Medical Genetics, Pediatric, and Radiology Services agreed on a diagnosis of Rothmund's syndrome. Skin fibroblast cultures were established at age 34 months.

RESULTS

From 100 subjects, 102 mass cultures of skin (epidermis and dermis of the mesial aspect of the mid-upper arm) were studied. The data shown in Figure 1 indicate that the longevity of a culture is a function of the age of the skin donor. Statistical analysis of this data (excluding cultures from fetuses and from patients with Werner's syndrome, Rothmund's syndrome, and progeria) indicates a regression of growth potential of the age of the donor from the 1st to the 9th decades. For the 1st to the 9th decades, the regression coefficient is −0.20 cell doublings per year, with a standard deviation of 0.05 and a correlation coefficient of −0.50. This regression coefficient is significantly different from 0 ($p < 0.01$). The average growth potential of approximately 44 cell doublings (Fig. 1), realized with cultures from fetal skin (from donors ranging in fetal age from 65 to 168 days), appears to be in good agreement with the results of Hayflick with the use of the fetal lung.[9] However, for various reasons noted in the Materials and Methods and Discussion sections of this paper, these data are

not strictly comparable, and in any case represent only minimal estimates of the growth potential of such cells.

When a single skin biopsy from a normal 35-year-old man was divided and used to establish duplicate mass cultures, the results were nearly identical (34.0 and 35.0 cell doublings). However, when two independent biopsies, one from the left arm and another from the right arm, were established from the same individual (a 6-month-old female with Down's syndrome and an unbalanced G/D translocation), somewhat different results were obtained (37.6 and 30.7 cell doublings). On the other hand, when the numbers of cumulative cell doublings were calculated for 59 clones independently derived from an established culture of human newborn foreskin fibroblasts, the results were remarkably consistent, with a mean of 63.1 cell doublings and a standard deviation of 7.6.[20]

Several biopsies were also obtained at necropsy from skeletal muscle (psoas) and lumbar vertebral bone marrow spicules, and in several cases the cumulative number of cell doublings was compared with that obtained with skin explants from the same individual. Cultures derived from the skin achieved the greatest number of cell doublings, and bone marrow spicules yielded the least, with skeletal muscle giving intermediate results (Fig. 2).

In addition, established mass fibroblast culture from

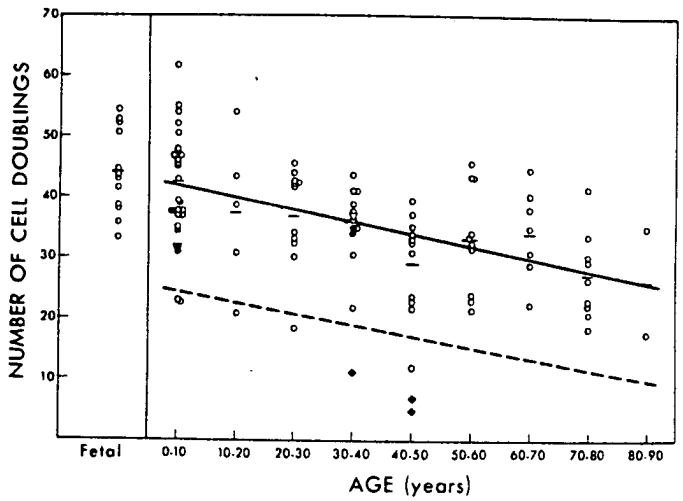

FIG. 1. The cumulative number of cell doublings achieved by human skin fibroblast cultures plotted as a function of the age of the donor. *Open circles*, Control cultures (see text); *closed diamonds*, Werner's syndrome homozygotes; *closed triangle*, Hutchinson-Gilford syndrome homozygote; *open triangle*, Hutchinson-Gilford syndrome heterozygote; *closed square*, Rothmund's syndrome homozygote; *closed circles*, parallel cultures from the same donor (left and right arm biopsies from a 6-month-old girl and bisected single biopsy from a 35-year-old man). The mean for each group is indicated by a *horizontal bar*. The calculated linear regression line (*solid line*) for the control group is drawn between the 1st and 9th decades and has a regression coefficient of -0.20 ± 0.05 standard deviation cell doublings per year with correlation coefficient of -0.50. The *dashed line* is the lower 95 per cent confidence limit for the regression line. In addition to Werner's syndrome, diagnoses for patients whose cultures fell below this line were: congenital heart disease with anomalous pulmonary venous drainage (age 2 weeks), cystic fibrosis (age 2), diabetes mellitus (age 17), acute metabolic encephalopathy ("Darvon" toxicity) (age 27), and carcinoma of the colon (age 44).

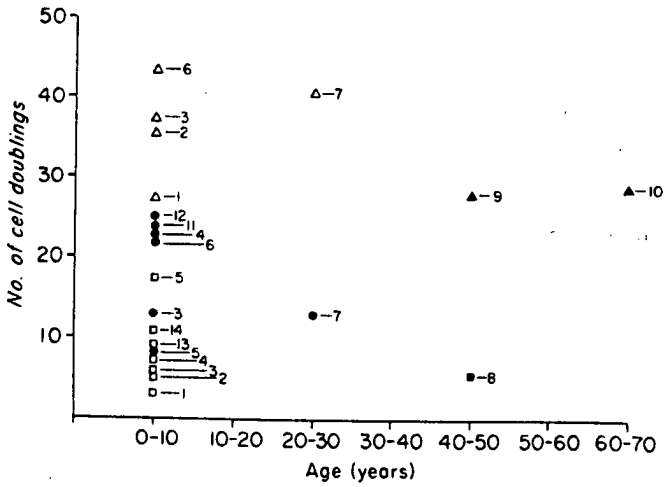

FIG. 2. The cumulative number of cell doublings achieved by human fibroblast cultures plotted as a function of the tissue of origin. *Open triangles*, skin (mesial aspect of midupper arm); *closed circles*, skeletal muscle (psoas); *open squares*, bone marrow spicules (lumbar vertebral); *closed triangles*, testis (controls); *closed square*, testis (Werner's syndrome). The numbers to the *right* of each culture identify the patients.

various regions of skin, subcutaneous fat, liver, kidney, or spleen of 40 other individuals and 150 skin fibroblast clones (including 68 secondary clones) have undergone "senescence" in our laboratory although the numbers of cell doublings achieved by these cultures are not known or were calculated on the basis of varying culture conditions. Thus, a finite replicative life-span appears to be an invariable characteristic of all cultures.

The original purpose of obtaining data for skin biopsies from individuals of various ages was to confirm our earlier report[3, 18] that a marked deficiency of *in vitro* growth potential is characteristic of fibroblasts derived from patients with Werner's syndrome. The present study (Fig. 1) demonstrates that the numbers of cell doublings for the skin fibroblast cultures from the two older patients with Werner's syndrome were more than two standard deviations below the mean of the distribution of control cultures for the 5th decade; the number of cell doublings was more than three standard deviations below the mean of the distribution of control cultures for the 4th decade in the case of our 37-year-old patient. The culture from the patient with progeria is less readily differentiated from the control cultures; however, it ranks number 23 of 26 cultures in this age group. Similarly, the culture from the patient with Rothmund's syndrome ranks 22 of 26 in that group. A fibroblast culture derived from the testis biopsy of the patient with Werner's syndrome and carcinoma of the prostate also had a sharply restricted life-span in comparison with control testis cultures derived from other patients with carcinoma of the prostate (Fig. 2).

In addition to the three cultures from patients with Werner's syndrome (which were not included in the regression analysis), five skin fibroblast cultures were found to fall below the lower 95 per cent confidence limit for the regression line (Fig. 1); the age and diag-

nosis of each of these patients is given in the legend to Figure 1.

DISCUSSION

Four major conclusions emerge from the present study.

Without exception, mass cultures and clones of somatic cells from a wide variety of human genomes and tissues eventually cease to replicate. Similar observations or observations interpreted as evidence of a progressive decline in growth rate have been made in other laboratories by using a wide variety of media and sera.[1, 4, 6-11, 14, 18, 21, 27, 28, 30, 33, 37] Therefore, it appears that one may now make the generalization that all cultures of normal somatic cells (normal in the sense that they carry the genome of the original host organism) have a finite replicative life-span *in vitro*. One of us[17] has referred to such cultures as "hyperplastoid" because they may serve as *in vitro* models of hyperplasia; they have also been referred to as "homonuclear" cell lines.[13] The prototype of the hyperplastoid cell line is the skin fibroblast culture—representing proliferating cells with a restricted growth potential, presumably a sampling of cells which undergo hyperplasia in a healing wound of the skin.

In contrast, cultures which appear to have an infinite growth potential have almost invariably been shown to carry genomes which differ from those of the original host organisms—even with crude cytogenetic techniques revealing only gross differences in chromosome number and karyotype.[13] One of us[17] has referred to such cultures as "neoplastoid" because they may serve as models for certain aspects of the neoplastic process; they have also been referred to as "heteronuclear." [13]

There are possible exceptions to the above generalization,[13] notably certain apparently immortal cell lines derived from the peripheral blood of chromosomally marked donors in which exogenous contamination by established neoplastoid cell lines[19] has been ruled out.[23] However, the techniques utilized in establishing such cultures, involving extremely large cell inoculums and prolonged latent periods,[22] suggest that these cultures derive from spontaneously or virally transformed cells.

The replicative life-span of cultured human somatic cells is a function of the tissue of origin (Fig. 2). Even in the case of explants obtained from the same individual, there is a far greater replicative life-span of cells derived from skin than from bone marrow, with cells derived from skeletal muscle yielding approximately intermediate results. The reasons for these differences are unknown, but presumably reflect differences in the previous *in vivo* history of the cells, such as their patterns of differentiation and their previous replicative history. It is therefore clear that interstrain comparisons must be based upon a standard site of biopsy. Thus, we have chosen the mesial aspect of the skin of the upper arm as our standard tissue because of (1) its convenience as a biopsy site, and (2) the relatively greater growth potential achieved by skin fibroblasts, permitting estimation of a wide range of cumulative cell doublings exhibited by various strains and providing greater numbers of cells for correlative biochemical and morphologic studies (none of which, however, are reported in the present communication).

The replicative life-span of cultured human skin fibroblasts is inversely related to the age of the donor (Fig. 1). The present series of cases, the largest so far published, confirms a similar conclusion made by others[7, 9-11] and leads to a calculated regression coefficient of -0.2 ± 0.05 standard deviation cell doublings per year from the 1st to the 9th decades, the first such data to be reported.

The replicative life-span of cultured human skin fibroblasts is a function of the genotype of the donor (Fig. 1). By using an independent biopsy and a greater number of controls, the present study confirms earlier work from this laboratory,[3, 18] indicating a striking decrease in growth potential of fibroblasts from a patient (H. Mc. G.) with Werner's syndrome. In addition, comparable results with biopsies from two other patients from different pedigrees now permit one to conclude that homozygosity for a single gene mutation can have a striking effect on the longevity of cultured somatic cells.

Goldstein[6] reported that skin fibroblasts from a 9-year-old boy with typical progeria could be subcultured only twice, whereas cultures from age-matched controls could be subcultured 20 to 30 times. Fibroblasts from our patient with progeria did not show such a striking impairment of growth potential; however, the fact that this culture ranked 23 of 26 in that age group suggests the possibility that this mutation may also result in diminished growth potential. Investigation of several additional patients is necessary to establish this point; it is possible, as is becoming increasingly evident for various other heritable disorders, that there are different types of progeria. The culture from the single patient with Rothmund's syndrome, the other recessively inherited disease which is sometimes confused with Werner's syndrome, ranks 23 of 26 cultures in that age group, and therefore this disorder may also be characterized by some degree of impairment of growth potential *in vitro;* nevertheless, the behavior of these cells *in vitro* is clearly different from that of skin fibroblasts derived from patients with Werner's syndrome. As regards the five "control" cultures which were found to fall below the lower 95 per cent confidence limit for the regression line (Fig. 1), the patient with diabetes mellitus may be of special interest in view of the recent publication of Goldstein *et al.*[7] Although these workers concluded that the life-spans of mass cultures from normals could not be differentiated from the life-spans of mass cultures from the progeny of conjugal diabetics, statistical analysis of their data (Fig. 1 of that paper, excluding their atypical case 6) by the *t*-test[20] indicates a significant difference ($p = 0.02$) between the means of the life-spans of cultures from prediabetic individuals over age 30 (mean = 49.1 ± 7.5 standard deviation cell doublings) and those from nondiabetics over age 30 (mean =

59.5 ± 5.2 standard deviation cell doublings). Their important discovery of a diminished cloning efficiency of fibroblasts from prediabetics, comparable to that which they observe in aging control cultures, suggests that the diabetic genotype may result in accelerated senescence, either *in vivo* or *in vitro*. Vracko and Benditt have evidence[34-36] that endothelial cells of diabetics may undergo increased numbers of replication *in vivo*. Studies with the alkaline phosphatase marker have suggested that a proportion of cells which migrate from a skin explant and participate in the establishment of the culture may in fact be endothelial cells.[24] It is possible that fibroblasts from diabetics also have a previous *in vivo* history of comparatively increased mitotic activity. In view of the finite growth potential of somatic cells, explants from diabetics, especially older diabetics, might then be expected to yield cells which have already utilized a significant proportion of their "allotted" number of cell doublings and hence display a more restricted growth potential *in vitro*. The same interpretation could of course be made in the case of Werner's syndrome; all of our patients gave evidence of diabetes, a characteristic feature of the disease.[3] However, the simplest explanation of our results with Werner's syndrome is that there is an intrinsic deficiency of growth potential as a result of homozygosity for the mutant gene.

Little can be made of the unusually low growth potential of the single case of cystic fibrosis (Fig. 1); additional cultures must be investigated with suitable controls. It is interesting, however, that cultured fibroblasts from patients with cystic fibrosis (as well as from a variety of other disorders) are characterized by abnormal accumulations of mucopolysaccharides.[2]

It should be emphasized that the absolute numbers of cell doublings reported in this and other[6-11, 32, 33] studies are merely estimates and are likely to vary from laboratory to laboratory depending upon the precise methods employed. First of all, there is no quantitative information with regard to the number of cells which migrate from a given explant and the number of cell doublings required to establish a culture. In the calculation of their data, Goldstein *et al.*[7] arbitrarily set at 10 the number of cell generations used up by the primary explants in attaining the first confluent monolayers; in the present study, this unknown number of cell doublings was not included in the calculations, and therefore comparatively lower figures for cumulative cell doublings are reported. Secondly, we have little information concerning interstrain variation in regard to the heterogeneity of the population of cells which do migrate from the explant and the extent to which they contribute to the population of mass cultures during the life histories of such cultures.[5, 16, 24-26] Furthermore, although we have indicated a plating efficiency (percentage of cells plated which stick to glass) of 75 to 90 per cent from randomly selected mass cultures of skin, it is possible that more substantial variations of plating efficiency occurred with cultures from tissues other than skin or from certain strains of skin fibroblasts. Finally, it is clear from the results of Todaro and Green[32] that media composition can influence the longevity of cultured somatic cells. Additional studies are required to determine the extent to which such factors may contribute to strain variations, particularly as a function of the tissue of origin, inasmuch as such factors as cellular heterogeneity, plating efficiency, and optimal media requirements might be expected to vary significantly from tissue to tissue.

In conclusion, we believe that our results, particularly with the skin fibroblast cultures, strengthen the hypothesis that human hyperplastoid cell lines[17] may serve as models for the study of senescence at the cellular level,[9-11] or more specifically, that aspect of senescence manifested by the loss of the mitotic potential of somatic cells. It is the challenge for the future to determine whether this senescence is specifically programmed by the genome or, alternatively, can be attributable to stochastic events (such as somatic cell mutation) which are merely modulated by the genome.

Note Added in Proof. By using the methods described in this paper, a fourth patient with Werner's syndrome (case 3 of Reference 3; biopsy of skin of distal ventral forearm obtained at age 43) yielded only 4.5 cell doublings. G. S. Merz and J. D. Ross. (J. Cell Physiol. 74:219, 1969).

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